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Disk Diffusion Susceptibility Testing of *Branhamella catarrhalis* with Ampicillin and Seven Other Antimicrobial Agents

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A total of 74 clinical isolates of *Branhamella catarrhalis* were characterized with respect to their ampicillin, amoxicillin-clavulanate, cephalothin, cefaclor, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole MICs and zones of inhibition. Disk diffusion tests were performed according to the guidelines of the National Committee for Clinical Laboratory Standards with two different media (Mueller-Hinton agar and chocolate Mueller-Hinton agar) and plates incubated under two atmospheric conditions (ambient air and 5 to 7% CO_2). Optimum disk diffusion test results were obtained with Mueller-Hinton agar plates incubated in ambient air with all eight antimicrobial agents. On the basis of comparisons of MICs versus zones of inhibition, the following zone diameter interpretive criteria were defined for testing *B. catarrhalis* with disks containing 10 μg of ampicillin: ≥38 mm, susceptible; 20 to 37 mm, moderately susceptible; ≤19 mm, resistant. The respective MIC correlates were ≤0.06, 0.125 to 0.5, and ≥1.0 μg/ml. Because of the absence of frankly resistant test organisms, it was not possible to make definitive recommendations pertaining to disk diffusion tests with amoxicillin-clavulanate, cephalothin, cefaclor, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. Evidence is presented, however, which suggests that the current National Committee for Clinical Laboratory Standards disk diffusion interpretive criteria for nonfastidious bacteria can be applied to *B. catarrhalis*, at least as they pertain to the susceptible category with cephalothin, amoxicillin-clavulanate, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. With cefaclor, a zone diameter of ≥21 mm was determined to adequately define the susceptible category.

Branhamella catarrhalis is now recognized as an important cause of acute otitis media, maxillary sinusitis, and bronchopulmonary infections in humans (4). Most clinical isolates of *B. catarrhalis* produce β-lactamase (1, 2, 6, 13, 15, 21). Infections caused by β-lactamase-producing strains have been reported to be refractory to therapy with β-lactam antimicrobial agents such as penicillin, ampicillin, and amoxicillin (17, 21, 24). Interestingly, however, there have been reports of patients with infections due to strains of *B. catarrhalis* which produced β-lactamase who have responded to treatment with these agents (5, 7, 12). Furthermore, several in vitro susceptibility studies have described β-lactamase-positive strains of *B. catarrhalis* with penicillin and ampicillin MICs as low as 0.1 to 0.5 μg/ml (3, 22, 23). It is possible, therefore, that the presence or absence of β-lactamase activity is not necessarily predictive of therapeutic outcome in all patients with *B. catarrhalis* infections who are treated with penicillin-class antimicrobial agents. If this were so, then some direct assay of in vitro activity such as a disk diffusion or broth-agar dilution test would be necessary to predict the therapeutic efficacy of these agents.

In addition, there have now been reports in Sweden, The Netherlands, and Japan of resistance to alternative agents such as trimethoprim-sulfamethoxazole, cefaclor, erythromycin, and tetracycline among clinical isolates of *B. catarrhalis* (9–11, 14, 20). Although resistance to these antimicrobial agents has not been reported in the United States, it is possible that resistance will become a problem. If this occurs, then there will exist a need for in vitro susceptibility tests which effectively predict the in vivo activities of these compounds as well.

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Heretofore, disk diffusion susceptibility testing of *B. catarrhalis* has not been systematically evaluated, at least with respect to antimicrobial agents other than penicillin and ampicillin. It was the intent of the current investigation to examine a standardized disk diffusion susceptibility procedure as a means for assessing the in vitro activities of ampicillin, amoxicillin-clavulanate, cephalothin, cefaclor, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole versus clinical isolates of *B. catarrhalis*.

MATERIALS AND METHODS

Organisms. Of 74 recently obtained clinical isolates of *B. catarrhalis* examined, 13 were from patients seen at the University of Massachusetts Medical Center, Worcester; 20 were provided by S. L. Berk, Veterans Administration Medical Center, Johnson City, Tenn.; 40 were the gift of R. J. Wallace, University of Texas Health Science Center, Tyler; and 1 was received from A. von Graevenitz, University of Zurich Institute of Medical Microbiology, Zurich, Switzerland. Of these 74 strains, 58 produced β-lactamase when tested in a conventional nitrocefin assay (19), and 16 lacked β-lactamase activity.

All strains were maintained as stock cultures at −70°C in 2% skim milk until just before testing. Stock cultures were thawed, and samples were inoculated onto plates containing 5% sheep blood agar (Scott Laboratories, Inc., Fiskeville, R.I.). Following incubation for 20 to 24 h at 35°C in ambient atmospheric air, isolated colonies were subcultured to a second sheep blood agar plate, which was incubated under identical conditions. Growth from this plate was used to prepare inocula for all subsequent studies.

TABLE 1. Comparisons of zone diameters obtained with 74 strains of *B. catarrhalis* tested with a standardized disk diffusion method by using two different test media and two different atmospheres of incubation

Antimicrobial agent	Avg (minimum/maximum) difference (mm) between zones of inhibition obtained under the following conditions:			
	MHA in CO_2 vs MHA in air	CHOC-MHA in CO_2 vs CHOC-MHA in air	CHOC-MHA in air vs MHA in air	CHOC-MHA in CO_2 vs MHA in CO_2
Ampicillin	+1.8 (−2/+8)	+1.3 (−2/+4)	−0.4 (−4/+1)	−0.9 (−5/+2)
Amoxicillin-clavulanate	+2.9 (−3/+10)	+0.6 (−4/+5)	−1.5 (−4/0)	−4.1 (−9/−1)
Cephalothin	+0.3 (−4/+7)	+0.1 (−4/+5)	−0.1 (−3/+1)	−0.4 (−6/+4)
Cefaclor	+5.4 (0/+13)	+3.2 (0/+8)	−1.4 (−4/+1)	−3.6 (−9/−2)
Erythromycin	+1.2 (−3/+6)	+0.6 (−3/+4)	−1.0 (−3/+2)	−1.7 (−5/+1)
Tetracycline	+12.2 (+6/+17)	+9.9 (+8/+14)	−7.2 (−10/−4)	−7.9 (−14/+2)
Chloramphenicol	+6.6 (+3/+13)	+2.9 (−1/+8)	−1.9 (−6/+1)	−5.6 (−11/−1)
Trimethoprim-sulfamethoxazole	+5.9 (+1/+12)	+5.1 (+2/+11)	−1.0 (−5/+2)	−1.7 (−6/+1)

Determination of MICs. MICs were determined by a tube broth macrodilution technique which used cation-supplemented Mueller-Hinton broth (pH 7.2; final volume, 2.0 ml) and an inoculum of 1×10^5 to 2×10^5 CFU/ml (final concentration). Mueller-Hinton broth was obtained from Scott Laboratories. The following antimicrobial agents, obtained as reagent grade powders from the manufacturers, were examined in twofold concentration increments ranging from 0.004 to 256 μg/ml: ampicillin (Bristol Laboratories, Syracuse, N.Y.), amoxicillin-clavulanate (Beecham Laboratories, Bristol, Tenn.), cephalothin (Eli Lilly & Co., Indianapolis, Ind.), cefaclor (Eli Lilly), erythromycin (Bristol), tetracycline (Parke, Davis & Co., Morris Plains, N.J.), chloramphenicol (Parke, Davis), and trimethoprim-sulfamethoxazole (Roche Diagnostics, Div. Hofmann-La Roche Inc., Nutley, N.J.). Amoxicillin-clavulanate was tested at a constant ratio of 2 parts amoxicillin to 1 part clavulanate; trimethoprim-sulfamethoxazole was tested at a constant ratio of 1 part trimethoprim to 19 parts sulfamethoxazole. Tubes were incubated at 35°C in air for 20 h and examined macroscopically for turbidity. The MIC was defined as the lowest concentration which yielded no macroscopic evidence of turbidity. All determinations were made in duplicate, and the results were averaged to obtain an estimate of the MIC for a given organism-antimicrobial agent combination. In cases in which duplicate MICs varied by only one twofold concentration, the lower value was assigned as the MIC for the organism. In no case were duplicate MIC determinations found to vary by more than fourfold.

Disk diffusion susceptibility procedure. A standardized disk diffusion susceptibility test was performed precisely as described by the National Committee for Clinical Laboratory Standards (16). Filter disks (BBL Microbiology Systems, Cockeysville, Md.) contained the following antimicrobial agents in the following amounts: ampicillin (10 μg), amoxicillin-clavulanate (20 or 10 μg), cephalothin (30 μg), erythromycin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg), and trimethoprim-sulfamethoxazole (1.25 or 23.75 μg). Cefaclor disks (30 μg) were obtained from Eli Lilly. Two different media were evaluated: Mueller-Hinton agar (MHA; pH 7.2) and MHA containing a 1.0% IsoVitaleX supplement (BBL) and 1.0% bovine hemoglobin (CHOC-MHA; pH 7.2). MHA was obtained from Scott Laboratories. Following inoculation and application of antimicrobial disks, plates were incubated for 16 to 18 h at 35°C in each of two atmospheres: ambient room air and 5 to 7% CO_2. Zones of inhibition were measured to the nearest millimeter with a caliper. All determinations were done in duplicate, and the results were averaged. In no case was a variation of >4 mm observed between the results of duplicate determinations.

RESULTS

The effects of medium composition and atmosphere of incubation on zones of inhibition obtained in disk diffusion susceptibility tests with *B. catarrhalis* are shown in Table 1. When test plates were incubated in both ambient atmospheric air and 5 to 7% CO_2, zones of inhibition were smaller on CHOC-MHA than on MHA. These differences were most conspicuous with selected antimicrobial agents, i.e., amoxicillin-clavulanate, cefaclor, tetracycline, and chloramphenicol. The atmosphere of incubation also seemed to influence zone sizes. Irrespective of the medium used, zones of inhibition obtained with disk diffusion plates incubated in 5 to 7% CO_2 were larger than those obtained with plates incubated in ambient air. Again, the differences were most pronounced with certain agents, in particular, cefaclor, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. The results of disk diffusion susceptibility tests performed with MHA plates incubated in ambient air were used for all further data analysis.

The relationship between MICs and zones of inhibition obtained with individual isolates of *B. catarrhalis* versus eight antimicrobial agents is depicted in Fig. 1 (ampicillin, cephalothin, cefaclor, and amoxicillin-clavulanate) and 2 (erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole). An inverse relationship between MICs and zones of inhibition was apparent with ampicillin and cephalothin and to some extent with cefaclor and amoxicillin-clavulanate. With erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole, there was no evident relationship between MICs and zones of inhibition; i.e., different strains with the same MICs had widely varying zone sizes.

It should be emphasized that, with the exception of ampicillin, the range of MICs for the 74 test strains was relatively narrow. Furthermore, on the basis of MIC determinations, none of the strains of *B. catarrhalis* examined in this study would have been characterized as frankly resistant to agents other than ampicillin.

β-Lactamase production appeared to influence MICs and zones of inhibition with three antimicrobial agents: ampicillin, cephalothin, and cefaclor (Table 2). Specifically, β-lactamase-negative strains of *B. catarrhalis* were inhibited by significantly lower concentrations of these three antimicrobial agents and had significantly larger zone sizes than did β-lactamase-producing strains. With amoxicillin-clavulanate, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole, the MICs and zone sizes for β-lactamase-producing strains were essentially equivalent to those obtained with strains which lacked β-lactamase.

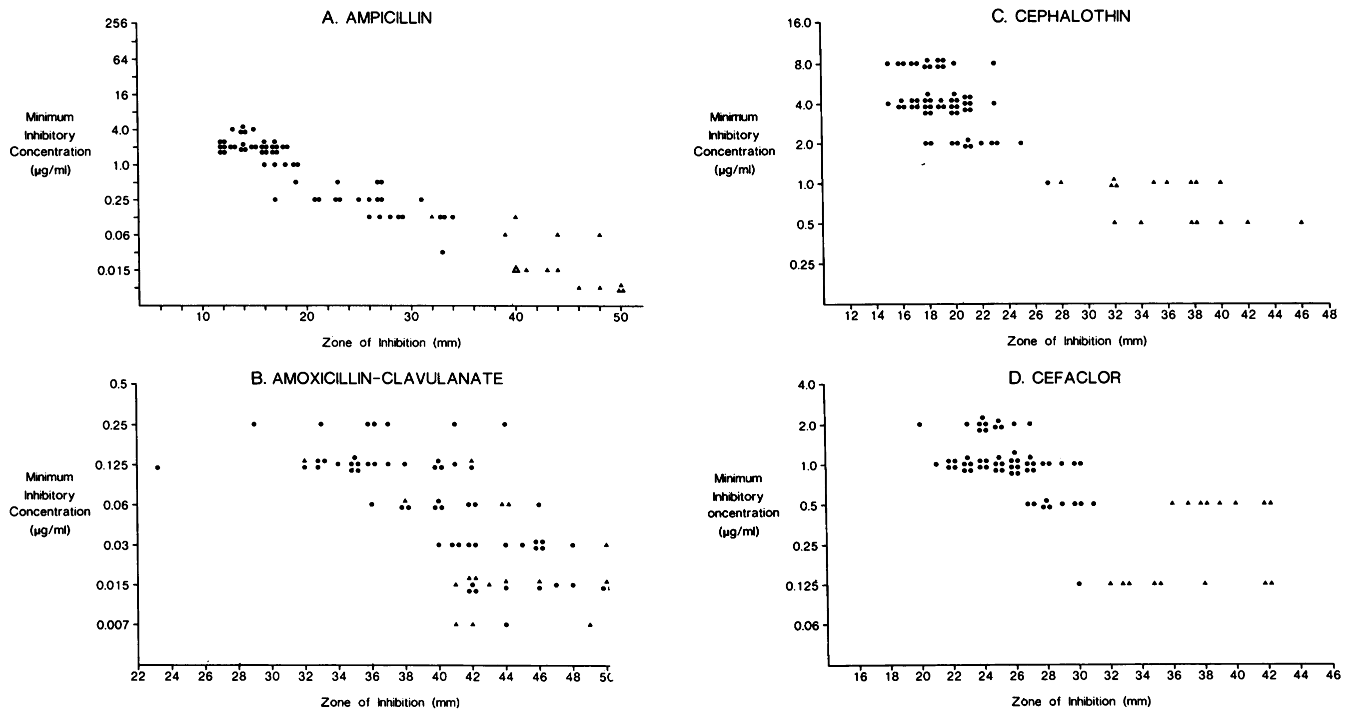

FIG. 1. Comparison of ampicillin (A), amoxicillin-clavulanate (B), cephalothin (C), and cefaclor (D) MICs and zones of inhibition for 74 strains of *B. catarrhalis*. Amoxicillin-clavulanate was tested at a constant ratio of 2 parts amoxicillin to 1 part clavulanate; the concentration listed is that of amoxicillin. Symbols: ●, β-lactamase-positive strains (n = 58); ▲, β-lactamase-negative strains (n = 16).

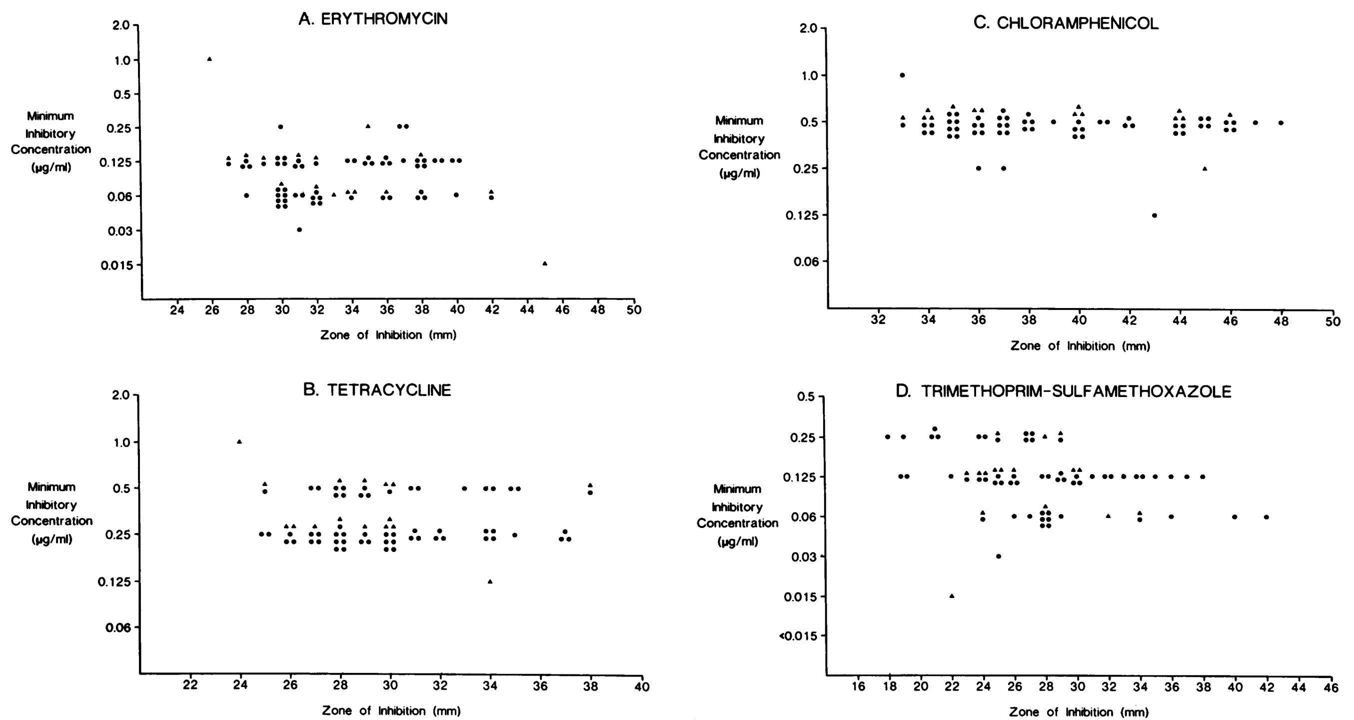

FIG. 2. Comparison of erythromycin (A), tetracycline (B), chloramphenicol (C), and trimethoprim-sulfamethoxazole (D) MICs and zones of inhibition for 74 strains of *B. catarrhalis*. Trimethoprim-sulfamethoxazole was tested at a constant ratio of 1 part trimethoprim to 19 parts sulfamethoxazole; the concentration listed is that of trimethoprim. Symbols: ●, β-lactamase-positive strains (n = 58); ▲, β-lactamase-negative strains (n = 16).

TABLE 2. Comparison of MICs and zones of inhibition obtained with β-lactamase-positive strains of *B. catarrhalis* versus strains which lacked β-lactamase[a]

Antimicrobial agent	Geometric mean MIC (μg/ml)		Avg zone diam (mm)	
	β-Lactamase positive	β-Lactamase negative	β-Lactamase positive	β-Lactamase negative
Ampicillin	0.84	0.03	19.6	43.3
Amoxicillin-clavulanate	0.06	0.02	39.9	43.1
Cephalothin	4.07	0.78	19.2	36.3
Cefaclor	1.0	0.25	25.6	37.6
Erythromycin	0.10	0.10	33.0	31.2
Tetracycline	0.33	0.36	30.2	29.0
Chloramphenicol	0.49	0.48	37.6	36.6
Trimethoprim-sulfamethoxazole	0.12	0.11	27.8	26.4

[a] MICs were determined with a tube broth macrodilution method. Zones of inhibition were determined by a standardized disk diffusion method which used Mueller-Hinton agar plates incubated in ambient air. We examined 58 β-lactamase-positive and 16 β-lactamase-negative strains.

DISCUSSION

In a recent communication which described the results of a nationwide laboratory proficiency survey pertaining to disk diffusion susceptibility testing of *B. catarrhalis*, significant variation was noted among results obtained from numerous participating laboratories (8). Since there existed little uniformity as to test method or interpretive criteria, this variability of test results was not unexpected. Indeed, the authors of this report emphasized the need for a systematic investigation of disk diffusion susceptibility testing of *B. catarrhalis* with the aim of clearly defining an optimum test method and suitable interpretive criteria. Recently, there have been two published studies which have addressed this issue (13, 23). In one study, however, only 25 strains of *B. catarrhalis* were examined, and 15 of these were not clinical isolates (23). In the second study, which incorporated a large number of clinically significant isolates of *B. catarrhalis*, the results of disk diffusion susceptibility tests were compared as to the presence or absence of β-lactamase production but not to quantitative estimates of antimicrobial activity (13). Recognizing the emerging significance of *B. catarrhalis* as a human pathogen (4) and the need for acceptable susceptibility test criteria (8), we conducted the current study in an attempt to define suitable conditions for performing disk diffusion tests with this organism and to define interpretive criteria.

The results of this investigation clearly indicate that medium composition and atmosphere of incubation influence the results of disk diffusion susceptibility tests with *B. catarrhalis*. With respect to medium composition, use of CHOC-MHA resulted in zones of inhibition that were, in general, smaller than those obtained with MHA. Incubation of test plates in an atmosphere of 5 to 7% CO_2 yielded zone sizes larger than those observed with plates incubated in ambient air. Because the current standard method for disk diffusion susceptibility testing of nonfastidious bacteria advocates use of MHA with incubation of test plates in air (16), and since all of the strains of *B. catarrhalis* examined in this study were readily propagated under these conditions, we advocate that disk diffusion susceptibility tests of *B. catarrhalis* be performed on MHA with plates incubated in ambient atmospheric air.

A recent report which characterized 231 clinical isolates of *B. catarrhalis* described 26 strains (11%) which failed to grow adequately on MHA in ambient air (13). As stated above, this problem was not encountered among the 74 strains characterized in the current study. Furthermore, we examined approximately 100 additional clinical isolates of *B. catarrhalis* and did not identify a single strain which failed to grow adequately on MHA in ambient air at 35°C after incubation for 18 h.

The results of the current investigation seem to provide a basis for making definitive recommendations regarding interpretive criteria to be applied when testing *B. catarrhalis* with 10-μg ampicillin disks. We propose that organisms for which MICs are ≤0.06 μg/ml be considered susceptible, those for which MICs are 0.125 to 0.5 μg/ml be identified as moderately susceptible, and those for which MICs are ≥1.0 μg/ml be viewed as resistant. The zone size interpretive criteria would be ≥38, 20 to 37, and ≤19 mm, respectively. These recommendations are based on definitions of susceptibility categories based on MIC ranges with extrapolation to zones of inhibition on the basis of linear regression analysis by the least-squares method. This approach seems to be justified, since a high degree of correlation was noted between MICs and zone diameters (i.e., $r = 0.864$). On the basis of the results of the current study, use of the zone size interpretive criteria recommended above would have resulted in the classification of one moderately susceptible (MIC, 0.125 μg/ml) strain as falsely susceptible, one susceptible (MIC, 0.03 μg/ml) strain as falsely moderately susceptible, and one moderately susceptible (MIC, 0.25 μg/ml) strain as falsely resistant.

Traditionally, β-lactamase production is construed as indicating penicillin and ampicillin resistance, irrespective of MICs or zones of inhibition. One consequence of using the interpretive criteria recommended above when performing ampicillin disk diffusion susceptibility tests with *B. catarrhalis* is that a small percentage of β-lactamase-producing strains is classified as moderately susceptible rather than resistant. We feel that this is justified for the following reasons. With respect to predicting therapeutic efficacy of ampicillin when used to treat *B. catarrhalis* infections, as stated in the introduction, MICs may be of greater predictive value than β-lactamase determinations insofar as not all infections due to β-lactamase-producing strains seem to be refractory to therapy with ampicillin. Further, organisms for which determination of β-lactamase production necessarily translates into clinical resistance to penicillin and ampicillin (i.e., *Staphylococcus aureus*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*) are not representative of *B. catarrhalis*. *B. catarrhalis* β-lactamase is distinctly different from the β-lactamases produced by these organisms. Finally, it should be recognized that a great deal is still to be learned about the role of *B. catarrhalis* as a human pathogen. When

we have a better understanding of this organism as a cause of human infection, it may be necessary to revise susceptibility test criteria.

It is difficult to make definitive recommendations regarding disk diffusion interpretive criteria for the other antimicrobial agents examined in this study, i.e., cephalothin, cefaclor, amoxicillin-clavulanate, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. First of all, strains of *B. catarrhalis* with frank resistance to these antimicrobial agents were not found. Secondly, with the exception of cephalothin and perhaps cefaclor and amoxicillin-clavulanate, an inverse relationship between MICs and zones of inhibition was not apparent. Despite these shortcomings, however, it may be possible to make certain assertions regarding *B. catarrhalis* disk diffusion susceptibility tests with these antimicrobial agents.

Specifically, it appears as though the zone diameter interpretive standards used to define the susceptible category in the National Committee for Clinical Laboratory Standards guidelines for disk diffusion testing of nonfastidious bacteria can be applied to *B. catarrhalis* when tested versus amoxicillin-clavulanate, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole (16). With respect to cephalothin disk diffusion testing of *B. catarrhalis*, the National Committee for Clinical Laboratory Standards zone size standards for both the susceptible and intermediate categories are acceptable.

Interpretation of the results of disk diffusion susceptibility testing of *B. catarrhalis* with cefaclor remains problematic. The National Committee for Clinical Laboratory Standards guidelines promulgate cephalothin as the class agent for certain related cephalosporins, including cefaclor (18). In the case of *B. catarrhalis*, the results of the current investigation clearly indicate that cefaclor and cephalothin differ considerably in their abilities to inhibit the growth of this organism, i.e., cefaclor possesses significantly greater activity than does cephalothin. Therefore, cephalothin cannot be used as a class agent for predicting the activity of cefaclor with *B. catarrhalis*.

The manufacturer of the cefaclor disks used in this study suggests use of the following zone diameter interpretive criteria: ≥21 mm, susceptible; 17 to 20 mm, intermediate; ≤16 mm, resistant. In the current investigation, all 74 study strains were susceptible to cefaclor on the basis of MICs, and zones of inhibition were ≥21 mm for all but one strain. The single exception was a strain for which the zone size was 20 mm. It can be inferred from these observations that the manufacturer recommended criteria for defining strains as susceptible to cefaclor probably apply to *B. catarrhalis*.

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